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Lairage during transport of eighteen-kilogram pigs has an impact on innate immunity and commensal bacteria diversity in the intestines^{1,2}

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ABSTRACT: Long distance transportation may affect the health of pigs; thus, adding a rest stop (lairage) during long journeys may improve their well-being. The objective of this study was to determine whether a mid-journey lairage influenced swine innate immunity and intestinal microbial populations after a 16-h transport. Four replications were conducted, 1 in each of 4 seasons. Eighteen-kilogram pigs were housed in 16 pens (13 to 16 pigs/pen) with 8 pens/treatment. Lairage pigs were transported for 8 h, given a rest with food and water for 8 h, then transported for an additional 8 h. Continuous pigs were continuously transported for 16 h. Jugular blood samples and intestinal tissue and contents were collected from 16 pigs (8/treatment) on d 1, 3, 7, and 14 posttransport. Hematocrit and white blood cell counts were determined and neutrophil cell functions, including phagocytosis/oxidative burst and phagocytosis of latex beads and leukocyte phenotypic cell markers (CD14 and CD18), were analyzed using flow cytometry. Expression of toll-like receptors 2, 4, and 5; IL-8 (a cytokine that is a chemoattractant for neutrophils); CCL20 (a chemokine that is a chemoattractant for dendritic cells); and the antimicrobial peptide PR39 were

determined from ileal and jejunal total RNA. Denaturing gradient gel electrophoresis was used to determine shifts in intestinal microbial populations. Total white blood cell and granulocyte counts in continuous pigs were greater ($P < 0.01$) on d 1 than in lairage pigs. Phagocytosis of microbeads was greater in continuous ($P < 0.05$) than in lairage pigs on d 7. Expression of IL-8 in jejunum was greater ($P < 0.05$) for continuous than for lairage pigs on d 1. Expression of CCL20 in the ileum was greater ($P < 0.05$) on d 14 for the continuous pigs. Expression of PR39 was greatest ($P < 0.05$) in the jejunum of lairage pigs on d 3. Lairage pigs had a greater ($P < 0.05$) variation in microbial populations (lower similarity coefficient) in the jejunum contents on d 1 and 7, in the cecum contents and tissue on d 3, and in the jejunum contents and tissue on d 14. However, continuous pigs had greater ($P < 0.05$) variation in the ileal tissues on d 14. This study indicates that adding a lairage to an extended transport alters immune functions, receptor, cytokine and chemokine expression, and gut microbiota compared with pigs transported for 16 h without lairage.

Key words: commensal bacteria, innate immunity, lairage, *Salmonella*, swine, transport

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INTRODUCTION

Transportation is an increasingly common and inevitable stressor in the swine industry. Nearly all animals

raised for food production are transported at least once, and some several times in their life span. Improvements in road quality and commercial pressures have led to increases in journey length, thus increasing the time of exposure to stressful conditions (Warriss, 2004). Transportation causes disruptions in daily time budgets, food and water withdrawal, exposure to novel environments and animals, extreme temperatures, vehicle noise and vibrations, close confinement, and predisposition to disease (Broom, 2005). By reducing stress, many biological processes improve (diverse microbiota, lower cortisol concentrations, improved immune responsiveness, greater feed intake, and growth) yielding enhanced well-being and healthier animals.

Susceptibility to infection is a concern because stress suppresses several measures of immune system func-

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²Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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tions of pigs (Warriss, 2004). In contrast, there are also hyper immune responses. Increased circulating white blood cell (WBC) counts, granulocyte numbers, and natural killer cells, which may optimize the immune system's ability to cope with pathogens and tissue damage sustained during a stressful event, are often demonstrated. Additionally, acute stress can also quickly alter microbial population variability in the gastrointestinal tract (Bailey et al., 1999; Soderholm et al., 2002; Richlin et al., 2004). Although these factors together may impair a stressed animal's health and viability, there is little research on effects of transportation stress regarding livestock species.

Therefore, the objective of this study was to evaluate the immunologic and intestinal microbial population alterations in swine with and without an 8-h lairage with access to food and water during a 16-h transport.

MATERIALS AND METHODS

Animals

All animal use procedures were approved by Purdue Animal Care and Use Committee.

A total of 894 terminal crossbred piglets (Yorkshire \times Landrace maternal line females bred to terminal sire Hampshire \times Duroc boars), born to sows maintained at the Purdue University's Animal Science Research and Education Swine Unit, were used in 4 replicates of the experiment. All piglets were born to sows housed in conventional farrowing crates with perforated metal flooring and were kept in their intact litters until weaning at an average of 19 d of age. At weaning, piglets were transferred to the same nursery where they were blocked by sex, BW, and litter into 16 identical pens (1.52×2.74 m) with perforated metal floors. Pens of pigs were randomly assigned to 1 of 2 transport treatments in a randomized complete block design. Pen was the experimental unit, with pig within pen as a random effect. Eight pens contained the lairage treatment and 8 pens housed the continuously transported treatment. After weaning, all piglets were fed, ad libitum, a nursery meal diet consisting of 22% CP for the first week, with 50 g/t of Carbodox, a pig starter transition feed consisting of 21% CP for the following 10 d, then a pig starter feed with 21% CP and 25 g/t of Carbodox (as fed) and allowed access to water at all times. There were 12 to 16 piglets per pen depending on animal availability during a particular replicate, with the number of pigs per pen balanced across treatments. Between 169 and 247 piglets were used per replicate, again depending on availability. Four weeks after weaning, the piglets were loaded onto the transport trailer.

Experimental Design

Four replicates were conducted, one each in January, April, August, and October (using 169, 247, 231, and 247 piglets, respectively), each a random season effect.

On a transport day, piglets were walked down an alley (length of the walk was between 2 and 12 m) from their nursery pens to a hydraulic lift trailer that moved them to the transport trailer. They were hauled on the lower level of a dual level, standard industry, transport trailer with pine shavings, and at a stocking density of 0.125 m²/pig. Pens on the bottom level of the trailer were constructed to keep pigs in their nursery pen group. The first pen was 2.5 m from the front of the trailer and the last pens were about 1.5 m from the end of the truck through which the pigs were loaded. There was no mixing within or across pens or treatments during any phase of the transport process. Both treatments were transported for 16 h; however, the lairage treatment was allowed a midjourney, 8-h lairage (a rest stop).

For ease of loading, the lairage treatment was loaded into 8 randomly assigned pens at the rear of the transport trailer, furthest away from the cab. Temperature and relative humidity data were recorded at 30-min intervals (Figure 1) using HOBO States (Onset Computer Corporation, Bourne, MA) throughout the truck, with less than 1°C difference indicating that no environmental extremes existed throughout the truck, which could have confounded the treatments. To ascertain that no location effects may be confounding this design, the data were compared within treatments for possible location on the truck effects. Data did not vary by location on the trailer. They were transported for 8 h on a mapped journey containing both rural and highway routes to simulate a standard industry transport. At the end of the 8 h, the truck returned to the Purdue University Swine Unit and the pigs were off-loaded by pen, into eight 1.68×4.27 -m, concrete, slatted-floor pens in the first grower/finisher barn for an 8-h lairage. Pigs were then immediately offered the nursery diet ad libitum and water for 2 h (the same diet offered in the nursery was offered in lairage), after which the feeders were removed from the pens for the remaining 6 h of lairage. Piglets were allowed access only to water. Meanwhile, the continuously transported treatment was loaded by pen into 8 randomly assigned transport pens at the front of the trailer, closest to the cab, to begin their first 8-h leg of the transport over the same route. After the lairage treatment's rest stop and the first 8-h leg of the continuous treatment's transport was completed, the truck returned to the Purdue University Swine Unit to reload the lairage pigs into their original 8 pens for the remaining 8 h of the transport, over the same route. At the end of the transport, all pigs were off-loaded by pen into the second grower/finishing barn into 16 randomly assigned 2.13×4.27 -m, concrete, slatted-floor pens with ad libitum access to feed and water.

Sample Collection

Within each replicate, jugular blood samples and intestinal tissue and contents were collected from 16 pigs (8 per treatment and 1 per pen) on each of d 0 (immedi-

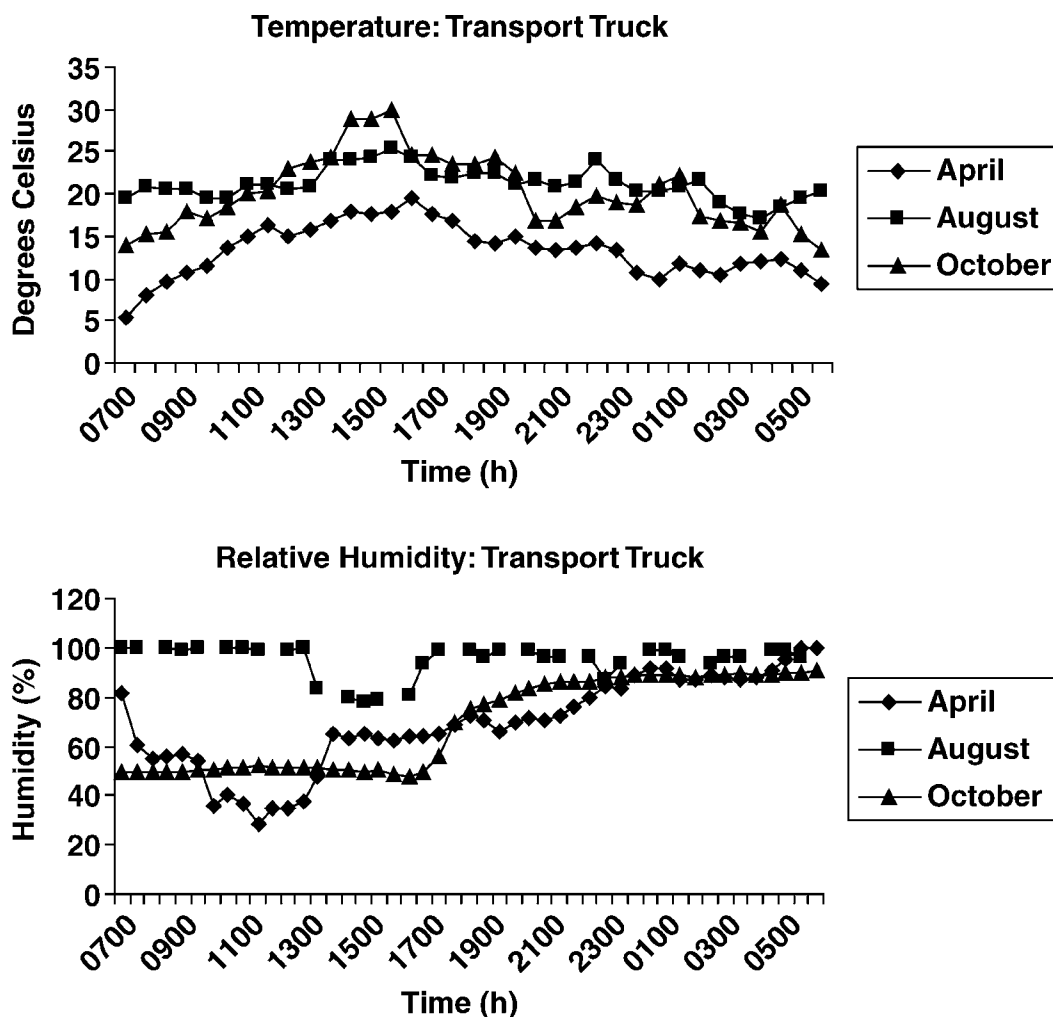


Figure 1. Mean temperature and relative humidity on the truck in various seasons. The January transport data were missed. The January environmental temperature began at 4.2°C and rose to 5.0°C by 1500 h and fell to 2.2°C by 0800 h on the return trip.

ately pretransport) and d 1, 3, 7, and 14 after transport. Pigs were anesthetized with 1 mL of a telazol-ketamine cocktail containing xylazine (Fort Dodge, Madison, NH). The telazol-ketamine cocktail was prepared using 500 mg of lyophilized Telazol reconstituted with 2.5 mL of ketamine (100 mg/mL) and 2.5 mL of xylazine (100 mg/mL). Then blood was collected via jugular venipuncture into vacuum tubes containing 10 mL of acid citrate dextrose (ACD; Becton Dickinson, Franklin Lakes, NJ) and 5 mL of EDTA (Becton Dickinson). Blood samples were placed on ice for transportation to the laboratory. Pigs were killed with 10 mL of i.v. Beuthanasia (Schering-Plough Animal Health Corporation, Summit, NJ) and exsanguinated before surgical incision sites were scrubbed with Operand iodine solution (Branford, CT). Collection of all samples was accomplished using sterile techniques.

Intestinal Contents and Tissue Collection

The ileocecal junction was located and mesenteric tissue was excised. Beginning at the ileocecal junction,

a cranial 4-m section along the length of the small intestine was marked; this indicated a representative and repeatable site for jejunal extraction. The jejunum was cut, and 2 mL of luminal contents was added to 10 mL of sterile buffered peptone broth (BPB; Difco Becton Dickinson Microbiology Systems, Sparks, MD) and 1 mL of jejunal luminal contents was collected into a 1.5-mL microcentrifuge tube. A 2 × 2-cm section of jejunal tissue was excised, cut along the mesenteric surface, rinsed with sterile 1× Hanks' Balanced Salt Solution (HBSS; Invitrogen Corporation, Carlsbad, CA), and placed into 1 mL of RNALater solution (Ambion Inc., Austin, TX) contained in a 1.5-mL cryotube, and another 2 × 2-cm section was placed in a 1.5-mL microcentrifuge tube. The ileum was cut 10 cm cranial to the ileocecal junction. Samples were collected as for the jejunum. From the apex of the cecum, a 2 × 2-cm section of tissue was collected, rinsed with sterile 1× HBSS, and placed into a 1.5-mL microcentrifuge tube. Two milliliters of cecal contents was then added to 10 mL of sterile BPB (Difco Becton Dickinson and Company),

Table 1. Primers and probes used for real-time PCR

Gene Name	Sequence, left primer (sense) 5'-3'	Sequence, right primer (antisense) 5'-3'
IL-8	GAGCTCTCCATCCAGCACAAG Probe: 6FAM-CCCCTGGGACAGCAGAGCTCACA-TAMRA	TCTCTTCCTGGCTCTGGTCCTA
CCL20 ¹	GCTCCTGGCTGCTTTGATG Probe: 6FAM-CGGTGTCTGTCTACCTCTGCA-TAMRA	CAGTCAAAGTTGCTTGCTTCTGA
TLR2 ²	CAGCACGAGAATACACAGTTTAACC Probe: 6FAM-ATTGGCTTCCCCAGACCCTGGAAGT-TAMRA	AACGAGTTGAGATTGTTATTGCTAATATCT
TLR4	TGTGGCCATCGCTGCTAAC Probe: 6FAM-TCATCCAGGAAGGTTTCCACAAAAGTCG-TAMRA	GGGACACCACGACAATAACCTT
TLR5	GTCTGCTCACCAGACAGACAA Probe: 6FAM-CTTCCTGCTGTCCACACGGCA-TAMRA	GATCGCATCAGCAACATCCA
PR39 ³	CGAGTCCCATCTCCCCACTT Probe: VIC-TCCTTGACCAGGACGAGGCCCG-MGBNFQ	CACCGTGAAGCTCACAGGTTT

¹CCL20 is a chemokine that attracts dendritic cells.

²TLR = toll-like receptor.

³PR39 is a proline-rich antimicrobial peptide.

and 1 mL of cecal contents was collected into a 1.5-mL microcentrifuge tube. Luminal and tissue samples, except luminal contents collected into BPB, were immediately placed on ice and then frozen (−80°C) until used for analysis.

Salmonella Detection

Jejunal, ileal, and cecal contents that had been diluted in 10 mL of BPB were incubated for 24 h at 37°C. One hundred microliters of the intestinal contents-BPB solution were then added to 3.0 mL of tetrathionate broth (Difco Becton Dickinson and Company) and incubated for an additional 48 h at 37°C. After incubation, 100 µL of the tetrathionate broth solution was added to 5 mL of Rappaport-Vassiliadis R10 Broth (Difco Laboratories, Detroit, MI) and was incubated for 24 h at 37°C. A 10-µL inoculation loop of Rappaport-Vassiliadis broth solution was then streaked onto XLT4 agar plates (Difco Becton Dickinson and Company) and allowed to incubate for 24 h. Black-colored bacterial colonies were transferred to triple sugar iron (TSI) agar slants (Becton Dickinson and Company, Cockeysville, MD), lysine iron agar (LIA) slants (Becton Dickinson Microbiology Systems), and Rambach agar plates (EMD Chemicals Inc., Gibbstown, NJ) using a 10-µL inoculation loop. Both the slant surface and the butt were inoculated for LIA and TSI media. *Salmonella* spp.-positive TSI slants appeared to have a yellow butt due to acid production, a black coloration on the slant and along the stab line, indicating hydrogen sulfide production, and an empty space under the agar indicating gas production. Slants of LIA converted from purple to a golden-black color in the presence of *Salmonella* spp. Additionally, Rambach agar turned red in the presence of *Salmonella* spp.

Intestinal Microbial Population Analysis

Jejunal, ileal, and cecal contents and tissues collected in 1.5-mL microfuge tubes were frozen at −80°C until assayed. Tissues were homogenized, and genomic DNA

was extracted from both tissues and contents using the MO BIO Laboratories Inc. UltraClean Fecal DNA Kit (Carlsbad, CA).

Polymerase chain reaction procedures were performed on the extracted DNA using bacterial-specific primers to conserved regions flanking the variable V3 region of 16S rDNA. Each PCR mixture contained 0.02 nmol of reverse primer (534r): 5'-ATT ACC GCG GCT GCT GG-3' and 0.02 nmol of forward primer with a GC clamp (341FGC): 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC CCC ACC CAG GAC-3' (Integrated DNA Technologies, Skokie, IL), 3.75 units of Taq DNA Polymerase, 1.0 µL of template DNA, 10 × DNA Polymerase Buffer (containing 10 mM Tris-HCL, 50 mM KCl, and 0.1% Triton X-100), and 25 mM MgCl₂ (Promega, Madison, WI). Amplifications were performed using a Hybaid PCR Express thermo cycler (Midwest Scientific, St. Louis, MO) using the following program: 1) denaturation at 94°C for 5 min; 2) subsequent denaturation at 92°C for 30 s; 3) annealing at 55°C for 30 s; 4) extension at 72°C for 30 s; 5) steps 2 to 4 repeated for 30 cycles; 6) subsequent extension at 72°C for 7 min; 7) 4°C final holding temperature.

Polyacrylamide gels [8% acrylamide:bisacrylamide ratio of 37.5:1 (vol/vol)] were cast to produce a 30 to 60% denaturing gradient. The 100% denaturing acrylamide used to create the gradient contained 7 M urea and 40% deionized formamide. The PCR-amplified samples were mixed with a 20% volume of 5 × loading buffer [0.025% (wt/vol) bromophenol blue, 0.025% (wt/vol) xylene cyanol, 47% (vol/vol) 0.1 M EDTA, and 47% (vol/vol) glycerol] and 20 µL was loaded into each sample well (20-well comb). Gels were placed in a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) and electrophoresed in 1 × Tris-acetate-EDTA buffer (VWR Scientific Products, Batavia, IL) at 60°C for 5 h and 30 min at 210 V. Gels were stained with a 1:5,000 (vol/vol) dilution of SYBR Green 1 nucleic acid stain (VWR Scientific Products) in 1× Tris-acetate-EDTA buffer for 15 min.

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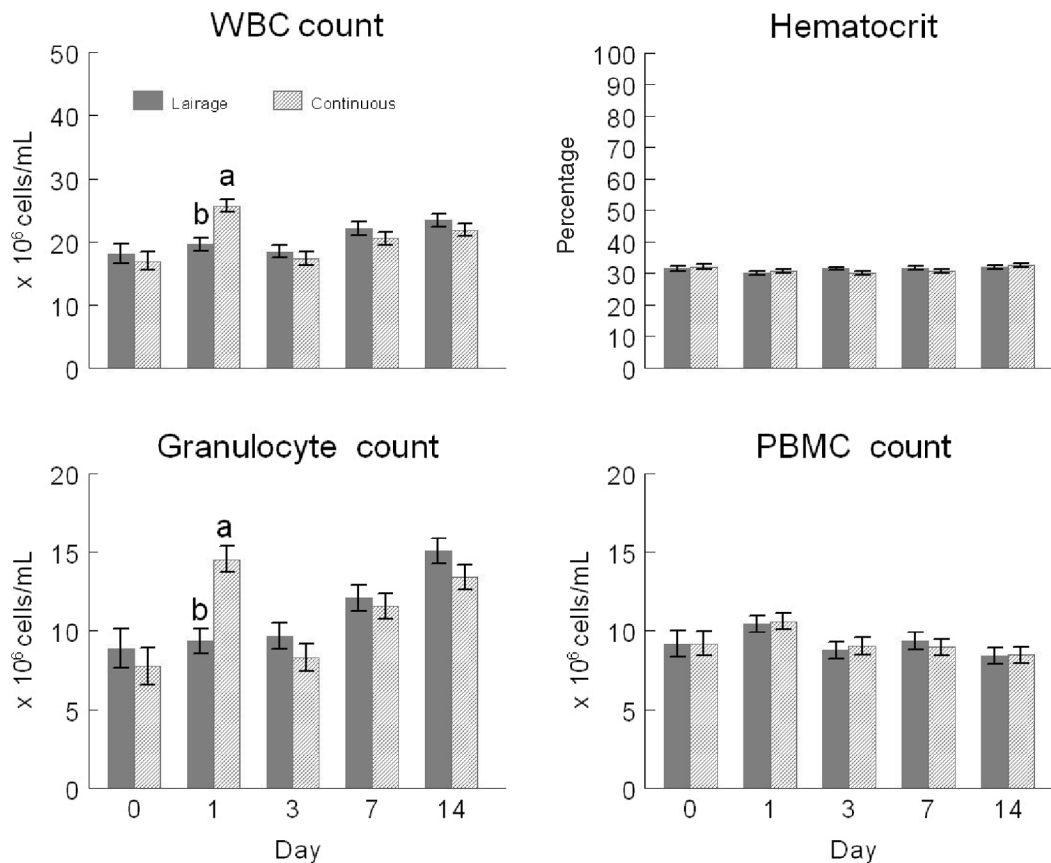


Figure 2. Total white blood cell (WBC), hematocrit, granulocyte, and peripheral blood mononuclear cell (PBMC) counts (least squares means \pm SE) from piglets offered a mid-transport lairage and piglets continuously transported. Time effect ($P < 0.01$) and time \times treatment interaction ($P < 0.01$) for WBC were found. Time effect ($P < 0.01$) and time \times treatment interaction ($P < 0.01$) were found for granulocyte counts. Peripheral blood mononuclear cell (lymphocyte and monocytes) count had a time effect ($P < 0.05$). ^{a,b}Means without common letters differ ($P < 0.01$).

Fragment pattern relatedness was determined using Bionumerics Software (Applied Maths, Austin, TX) version 2.5, which determined the number of bands per sample and a similarity index for bands within a sample. The number of bands is indicative of the number of predominant bacterial species present within a specific intestinal ecosystem. Similarity coefficients are based on the number and position of bands in pairwise comparisons. The within-treatment comparisons were used to determine how similar the gut bacterial population profiles were within the treatment sample population. The cross products, however, are comparisons between each individual sample and all the other samples of the opposite treatment, indicating population similarity resulting if there were no treatment effects. These similarity coefficients, both within treatment and across treatment (cross products), were then statistically analyzed using PROC MIXED (SAS Inst. Inc., Cary, NC) and treatment differences were determined using the Tukey-Kramer means separation method.

Peripheral Leukocyte Populations

White blood cell counts, granulocyte counts, and counts of the peripheral blood mononuclear cells

(PBMC; lymphocyte and monocytes) and percentages of each were assessed using the IDEXX VetTest 8008 (Westbrook, ME) for the first replication and the Hema-vet HV950FS (Oxford, CT) blood analyzing system for the remaining replications. The IDEXX system was used only for the first replication due to mechanical failure; however, both methods were validated via an unbiased, trained technician using a differential count.

Peripheral Leukocyte Flow Cytometric Analysis

All flow cytometry was conducted using the Coulter Epics XL-MCL Flow Cytometer and System II software (Beckman Coulter Inc., Miami, FL). The flow cytometer used a 488-nm, air-cooled argon laser for excitation, a 525-band pass filter for detection of fluorescein isothiocyanate (FITC) emissions and a 575-band pass filter for detection of phycoerythrin (PE) emission. For each sample, a total population of 10,000 cells was analyzed.

CD14 and CD18 Cell Surface Expression. Five hundred microliters of whole blood from the ACD tubes was incubated on ice for 30 min with 20 μ L of phycoerythrin-conjugated, monoclonal, mouse anti-human CD14, clone TUK4 (DakoCytomation, Glostrup, Denmark)

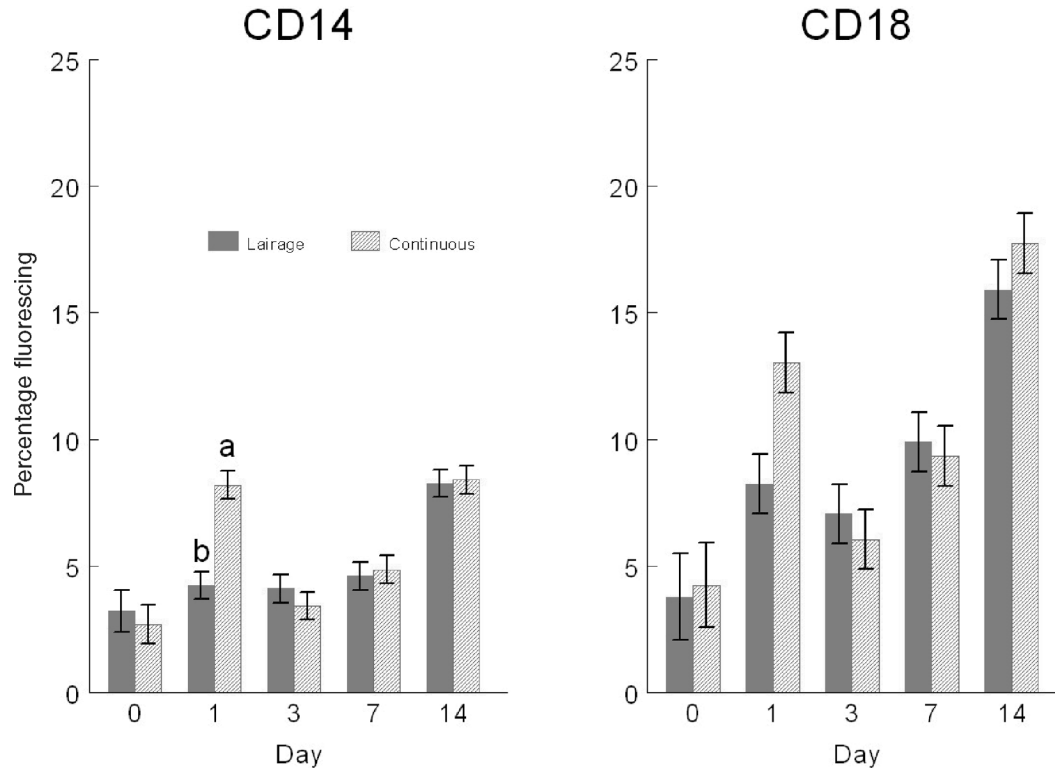


Figure 3. Percentage of peripheral blood leukocytes positive for CD14 and CD18 cell surface expression (least squares means \pm SE) from piglets offered a mid-transport lairage and piglets continuously transported. Time effect ($P < 0.01$) and time \times treatment interaction ($P = 0.01$) were found for CD14. The percentage of peripheral blood leukocytes positive for cell surface CD18 had a time effect ($P < 0.01$). ^{a,b}Means without common letters differ ($P < 0.01$).

and 20 μ L of monoclonal mouse anti-human LFA-1, β -chain/fluorescein isothiocyanate CD18, clone MHM23 antibodies (DakoCytomation; 100 mg/L). Another tube remained without antibody to serve as unstained control cells and allowed gating of auto fluorescence. After incubation, cells were lysed by hypotonic lysis, in which 900 μ L of cold sterile water was added to each sample tube for 30 s. After red blood cell lysis, 0.1 mL of 10 \times HBSS was added to restore isotonicity. Samples were washed with 1 mL of 1 \times HBSS and then centrifuged at 1,800 $\times g$ for another 5 min at 4°C. The cell pellet was resuspended in 1 mL of 1 \times HBSS for flow cytometric analysis.

Phagocytosis and Oxidative Burst. Dihydrorhodamine (DHR; Molecular Probes, Eugene, OR) was diluted in 1 \times HBSS to a 10 mg/mL working solution in a 15-mL conical tube and protected from light for later use. In addition, 50 μ L of Pansorbin (10% suspension of *Staphylococcus aureus* cells in PBS, 0.1% NaN₃, pH 7.2; Calbiochem, LaJolla, CA) was labeled with 50 μ L of propidium iodide (PI; Calbiochem) in a 5-mL polypropylene tube and incubated at 37°C for 30 min. The PI-labeled Pansorbin was then washed twice with 1 \times HBSS and centrifuged at 1,800 $\times g$ for 2 min at 4°C. The PI-labeled Pansorbin was resuspended in 100 μ L of 1 \times HBSS, and 0.25 μ L of *Staphylococcus aureus* BioParticles opsonizing reagent (Molecular Probes) was added. The solution was incubated at 37°C in a shaking

water bath for another 30 min. After incubation, opsonized PI-labeled Pansorbin was washed with 2 mL of 1 \times HBSS and centrifuged at 1,800 $\times g$ for 3 min at 4°C, and the cells were resuspended in 100 μ L of 1 \times HBSS for later use. Four hundred fifty microliters of whole blood from the ACD tubes was incubated in a shaking water bath at 37°C for 60 min. One hundred microliters of DHR was then added to each sample to indicate peroxidase and chemical oxidative activity, and the mixtures were incubated at 37°C for 10 min in a shaking water bath.

After incubation, 50- μ L aliquots of the blood/DHR solution were lysed by hypotonic lysis. Cells were washed once with 1 mL of 1 \times HBSS. The cell pellet was resuspended in 1 mL of 1 \times HBSS and placed on ice for further analysis. The remaining sample tube was inoculated with 50 μ L of opsonized PI-labeled Pansorbin and incubated at 37°C for 10 min in a shaking water bath. Red blood cells in samples were lysed, and the remaining cells were washed twice as described above. Samples were then placed on ice, protected from light, and analyzed by flow cytometry.

Microbead Phagocytosis. Four hundred fifty microliters of whole blood was taken from blood collected into the ACD vacutainer tubes and incubated in a shaking water bath for 60 min at 37°C. After incubation, a 50- μ L aliquot serving as a control was hypotonically lysed. The cell pellet was reconstituted with 1 mL of 1 \times HBSS

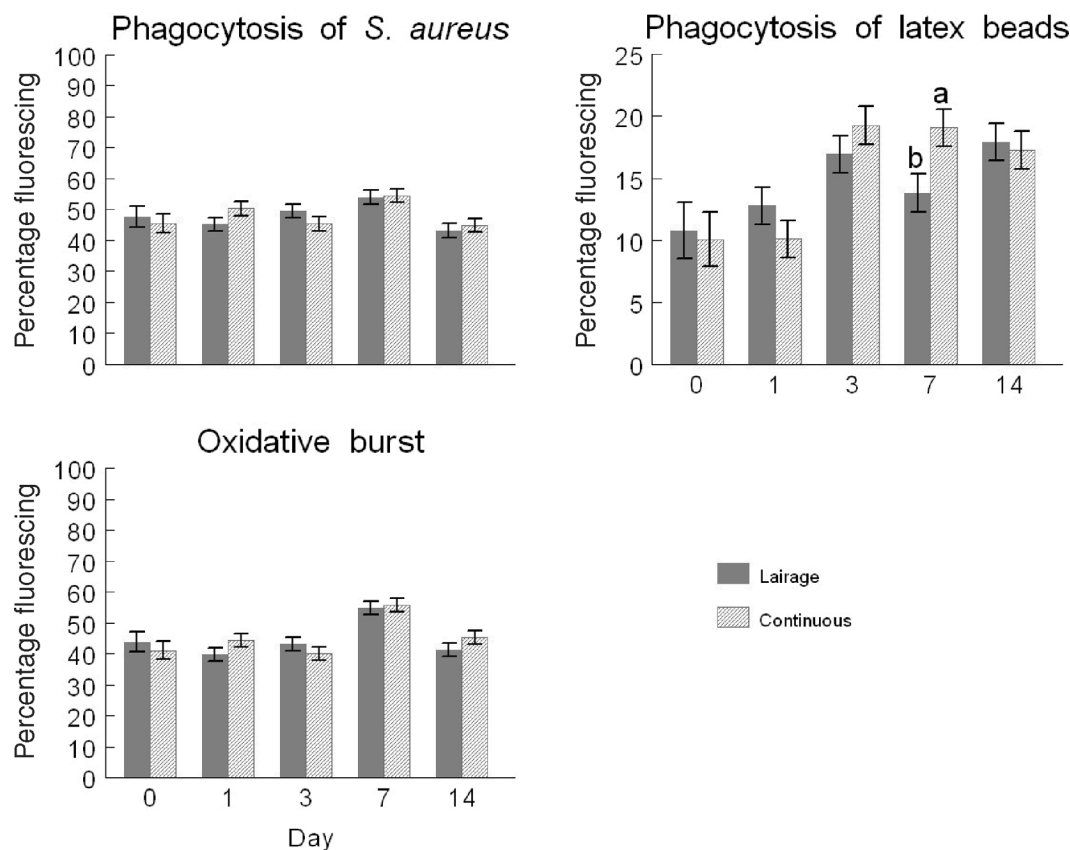


Figure 4. Percentage of peripheral cells positive (least squares means \pm SE) for phagocytic activity of *Staphylococcus aureus* or latex beads and oxidative burst following *S. aureus* ingestion by total leukocyte population from piglets offered a mid-transport lairage and piglets continuously transported. Time effects were found for *S. aureus* phagocytosis ($P < 0.05$), cells positive for oxidative burst activity ($P < 0.01$), and cells positive for phagocytizing latex microbeads had a time effect ($P < 0.01$). ^{a,b}Means without common letters differ ($P < 0.01$).

and placed on ice for later analysis. Twelve and one-half microliters of FluoSpheres carboxylate-modified microspheres (0.04 μ m; Molecular Probes) was added to the remaining 400 μ L and incubated in a shaking water bath at 37°C for 40 min. After incubation, a 50- μ L aliquot was removed and added to a 5-mL polypropylene tube. Red blood cells were lysed as described previously and washed with 1 \times HBSS, and the cells were resuspended in 1 mL of 1 \times HBSS and placed on ice for analysis and protected from light.

Intestinal Receptor, Cytokine, and Chemokine Expression

RNA Extraction. Extraction was performed on whole blood collected in the ACD vacuum tubes and homogenized ileal and jejunal tissue samples using QIAmp RNA Blood Mini Kit (Qiagen, Valencia, CA). Extracted RNA was quantified using the GeneQuant pro RNA/DNA Calculator (Amersham Biosciences Corp., Piscataway, NJ).

cDNA-Synthesis. All instruments and surfaces to be used were treated with RNase Zap (Ambion Inc.) before beginning reverse transcription. Master mix was composed of reagents included in the TaqMan Reverse

Transcription Reagent Pack (Applied Biosystems, Foster City, CA) and was prepared in a sterile disposable reagent reservoir (Vistalab Technologies, Mt. Kisco, NY) with random hexamers in a 100- μ L final volume containing 50 U/ μ L of Multi-Scribe reverse transcriptase, 25 mM MgCl₂, 2.5 μ M random hexamers, 0.4 U/ μ L RNase inhibitor, 50 μ M dNTPs, and TaqMan reverse transcription buffer (TaqMan reverse transcription reagents, Applied Biosystems). RNase-free water (Ambion Inc.) and sample RNA were added to the Master mix at appropriate quantities, then placed in the Hybaid PCR Express thermo cycler (Midwest Scientific, St. Louis, MO) and run using the following program: 1) 50°C for 2 min; 2) 95°C for 10 min; 3) 95°C for 15 s; 4) 60°C for 1 min; 5) steps 3 and 4 were repeated for 30 cycles; 6) the final stage was 60°C for 5 min with a holding temperature of 4°C. The resulting cDNA was stored at -80°C until further analysis.

Primers and Probes. Primers and probes (Table 1) used for real-time PCR were developed using Primer Express and synthesized by Applied Biosystems. One hundred microliters of a 10 μ M solution was aliquoted into ten 0.5-mL RNase-free tubes (Dot Scientific Inc., Burton, MI; Ambion Inc.) for future use to reduce the

occurrence of freezing and thawing. In addition, before each plate was prepared, stock probes were diluted to approximately 10 μ M solutions.

Quantitation of Genes of Interest Expression by Real-Time PCR: Intestinal Tissue. The abundance of the genes of interest relative to the quantity of 18S rRNA in total RNA isolated from homogenized jejunal and ileal tissue was quantified using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). To assure consistency of sample preparation and amplification, all samples were analyzed in triplicate on the same 96-well PCR reaction plate (Applied Biosystems) with assurance of contamination-free conditions due to use of a nuclease-free water control for each gene analyzed in duplicate. The reactions were carried out using the appropriate forward and reverse primers (900 nM), the appropriate TaqMan probe (200 nM), PCR Mastermix (Applied Biosystems), and 3.5 μ L of the cDNA sample and a program consisting of the following: 1) an initial step at 50°C for 2 min; 2) followed by 10-min step at 95°C; 3) then 20 s at 95°C; 4) 1 min at 60°C; 5) repeat steps 3 and 4 for 50 cycles. Commercially available eukaryotic 18S rRNA (Applied Biosystems) primers and probe were used as an endogenous control.

Statistical Analyses

Leukocyte population, flow cytometric, and real-time PCR data were checked for normality and appropriately transformed when necessary until normality was achieved. Data were analyzed using PROC MIXED of SAS, with day, time (sample time point; pretransport, posttransport, d 3, d 7, d 14), treatment and treatment \times time interaction as fixed effects and pig within pen and replication (season) as random variables. The appropriate covariate structures were used based on the Bayesian criterion; compound symmetry and simple for blood data and real-time PCR data, respectively. Data from denaturing gradient gel electrophoresis analysis were also analyzed with the MIXED procedures of SAS, separating means using the Tukey-Kramer adjustment and treatment as a fixed effect. Relative abundance of receptors, cytokines, and chemokines in jejunal and ileal samples were determined using the $\Delta\Delta$ CT method, with the average pretreatment Δ CT (d 0) as the reference.

RESULTS

Peripheral Blood

Hematocrit values were relatively stable and had no main effects or interactions (Figure 2). There was a time effect ($P < 0.01$) and a time \times treatment interaction ($P < 0.01$) for total WBC blood cell count (Figure 2). Continuously transported pigs had a greater ($P < 0.01$) WBC count than lairage pigs on d 1. The continuous pigs had fewer ($P < 0.05$) total WBC before transport than on all days except d 3. Day 1 WBC counts were

the greatest ($P < 0.05$) for continuous pigs. Day 14 WBC counts in continuous pigs were also greater ($P < 0.01$) than on d 3. In lairage pigs, d 7 and 14 WBC counts were greater ($P < 0.05$) than d 3, and d 14 counts were greater ($P < 0.05$) than those on d 1.

A time effect ($P < 0.01$) and a time \times treatment interaction ($P < 0.01$) were found for granulocyte counts (Figure 2). Granulocyte counts were greater ($P < 0.01$) on d 1 in continuous pigs than in lairage pigs. Control pigs had the greatest ($P < 0.01$) count on d 1 compared with all days except for d 14. Granulocyte counts on d 7 and 14 were greater ($P < 0.01$) than d 0 in continuous pigs, greater ($P < 0.05$) than d 3 in both treatments, and greater ($P < 0.01$) than d 0 and 1 in lairage pigs.

A time effect ($P = 0.01$) was observed for PBMC counts (Figure 2). In both treatments, d 1 was greater ($P < 0.05$) than d 14.

CD14 and CD18 Expression. A time effect ($P < 0.01$) and a time \times treatment effect ($P = 0.01$) were detected for percentage of cells positive for CD14 expression (Figure 3). On d 1, continuous pigs had a greater ($P < 0.01$) percentage of cells expressing CD14 than lairage pigs. In continuous pigs, d 0 percentage of cells positive for CD14 was less ($P < 0.01$) than all other days that were analyzed except for d 3. Also in continuous pigs, d 1 percentage was greater ($P < 0.01$) than both d 3 and 7 percentages, d 7 and 14 percentages were greater ($P < 0.01$) than d 3, and d 14 percentage was greater than that on d 7 ($P < 0.01$). Day 14 CD14 expression was greatest ($P < 0.01$) within lairage pigs.

A time effect ($P < 0.01$) was detected for percentage of cells expressing CD18 (Figure 3). Day 1, 7, and 14 percentages were greater ($P < 0.01$) than pretransport percentage, and d 1 and 14 percentages were greater ($P < 0.05$) than d 3 and 7 percentages in continuous pigs. In lairage pigs, d 7 and 14 percentages were greater ($P < 0.05$) than pretransport percentages, and d 14 percentage was greater ($P < 0.01$) than percentages on d 1, 3, and 7.

Phagocytosis and Oxidative Burst. A time effect ($P < 0.05$) was found for the percentage of cells positive for phagocytosis of *S. aureus* (Figure 4). In both treatments, the d 7 percentage was greater ($P < 0.05$) than d 14. A corresponding time effect ($P < 0.01$) for percentage of cells with fluorescence indicating oxidative burst was found (Figure 4). Percentage of cells positive for phagocytosis of the continuously transported pigs was greatest on d 7 ($P < 0.05$), and in lairage pigs d 7 was greater ($P < 0.01$) than all time points except pretransport.

A time effect ($P < 0.01$) was detected for the percentage of cells positive for microbead phagocytosis (Figure 4). Lairage pigs had less microbead ($P = 0.01$) phagocytosis on d 7 than continuous pigs. Day 3, 7, and 14 percentages of cells fluorescing were greater ($P < 0.01$) than d 0 and 1 in continuous pigs. In lairage pigs, d 3 and 14 had greater ($P < 0.01$) percentages than pretransport values.

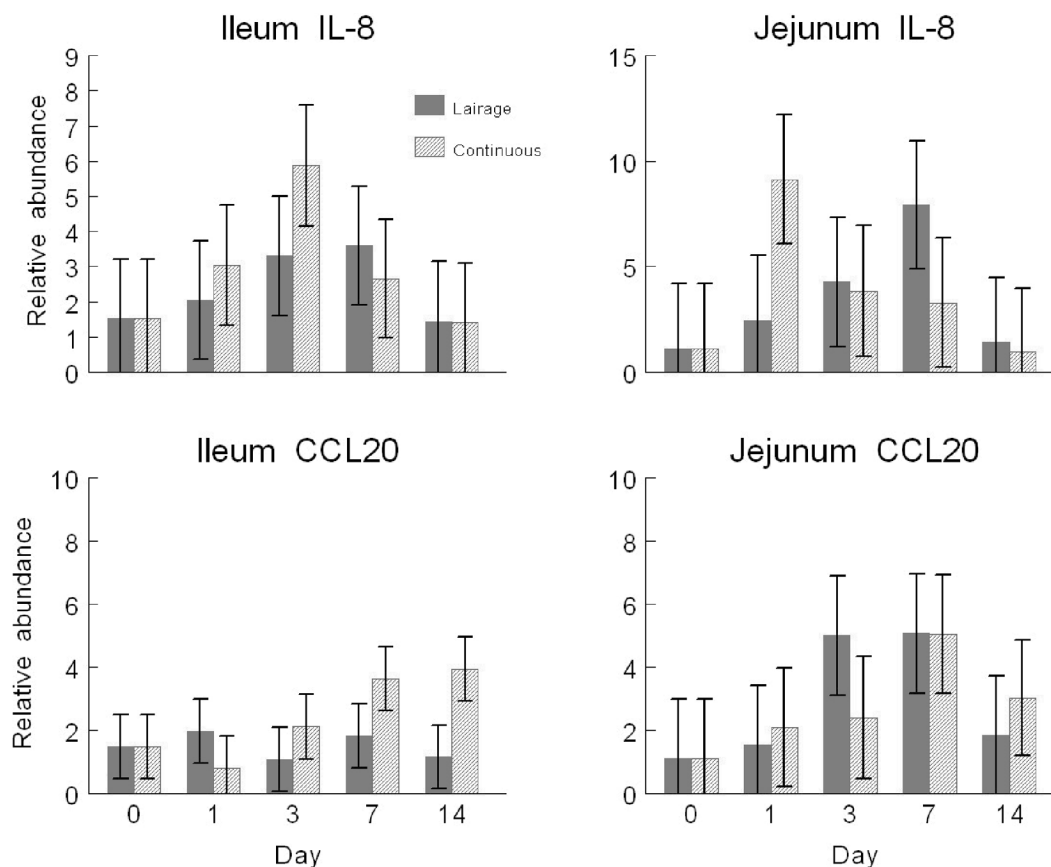


Figure 5. Relative abundance (least squares means \pm SE) of IL-8 (a chemoattractant for neutrophils) and CCL20 (a chemokine that is a chemoattractant for dendritic cells) from mRNA in ileal and jejunal tissues. Abundance of IL-8 or CCL20 was compared to 18S mRNA in the ileum or jejunum of piglets offered a mid-transport lairage and piglets continuously transported. Time effect was found for ileal ($P < 0.01$) and jejunal ($P < 0.001$) IL-8 expression. Jejunal CCL20 expression had a trend toward a time effect ($P < 0.10$).

Intestinal Tissue Cytokine, Chemokine, Receptor, and Peptide mRNA Relative Abundance

Chemoattractants, Interleukin-8, and CCL20. A time effect ($P < 0.01$) was observed, but no treatment effect or time \times treatment interaction were observed for relative abundance of IL-8 mRNA in jejunal tissues (Figure 5). Continuously transported pigs had a greater ($P < 0.05$) relative abundance of mRNA on d 1, 3, and 7 compared with d 14, and d 3 had greater ($P < 0.05$) abundance than pretransport values. In lairage pigs, d 14 had less ($P < 0.05$) relative abundance than d 3 and 7 and d 7 was also greater ($P < 0.05$) than before transport. A time effect ($P = 0.01$) was observed for relative abundance of IL-8 mRNA in ileal tissues (Figure 5). In continuous pigs, d 0 and 1 had a greater relative abundance ($P < 0.01$) than d 14, and d 3 was greater ($P < 0.01$) than both d 7 and 14. No main effects or interactions were detected for relative abundance of CCL20 in jejunal or ileal tissues (Figure 5).

Toll-like Receptors. Although there were no main effects or interactions for relative abundance of toll-like receptors (TLR) 2, 4, or 5 mRNA in ileal tissues (Figure 6), a time effect ($P < 0.01$) was detected but no treatment

effect or time \times treatment interactions were observed for relative abundance of TLR4 mRNA in jejunal tissues (Figure 7). A greater relative abundance of TLR4 mRNA ($P < 0.05$) was observed on d 3 compared with d 14 in continuous pigs. In lairage pigs, d 3 had the greatest relative abundance ($P < 0.05$) of TLR4 mRNA. However, no main effects or interactions for relative abundance of TLR2 or 5 mRNA were observed in jejunal tissues.

PR39. A time effect ($P < 0.01$) but no treatment effect or time \times treatment interaction for relative abundance of PR39 mRNA was detected in jejunal tissues (Figure 8). Lairage pigs, on d 3, had the greatest ($P < 0.05$) relative abundance of PR39 mRNA. On d 1 and 3 the relative abundance of PR39 in continuous pigs was greater ($P < 0.05$) than on d 14. No main effects or interactions for relative abundance of PR39 mRNA were found in ileal tissues (Figure 8).

Intestinal Microbial Populations

Few incidents of *Salmonella* were detected and occurred in both treatments (data not shown). Microbial populations of the lairage pigs were more ($P < 0.01$) varied (lower similarity coefficient) on d 1 in jejunum

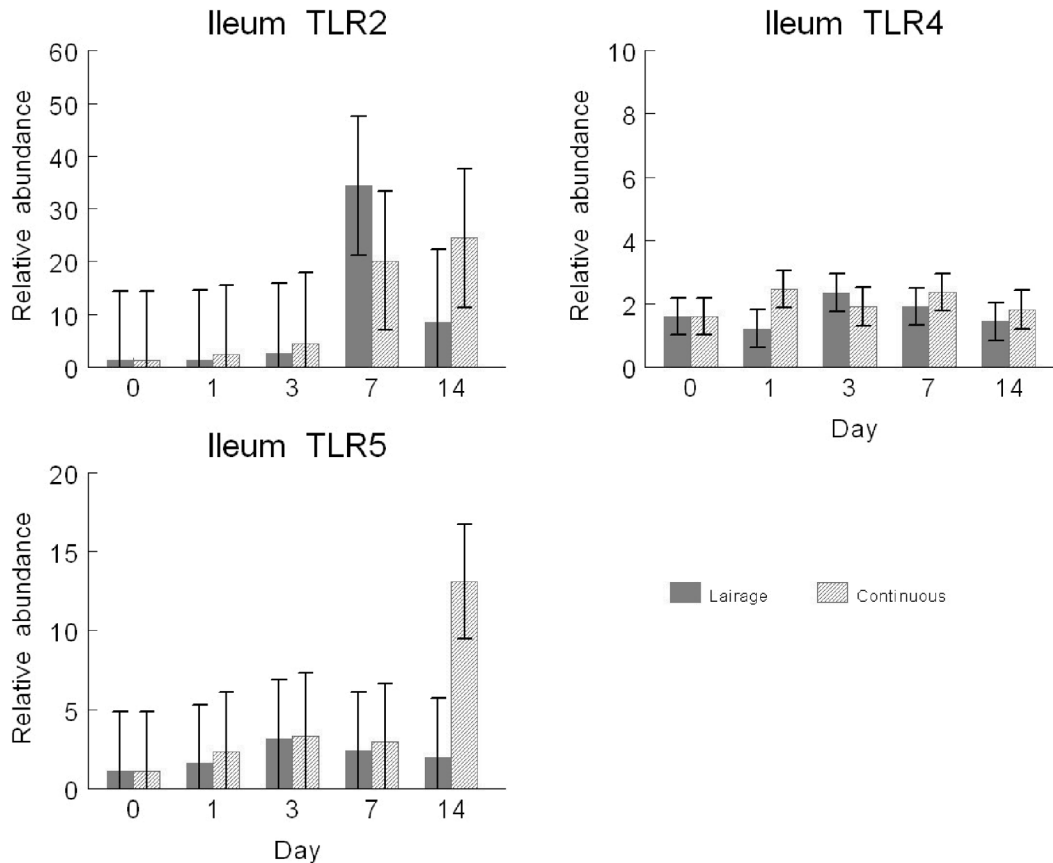


Figure 6. Relative abundance (least squares means \pm SE) of toll-like receptor (TLR) 2, TLR4, and TLR5 mRNA in ileal tissues. Abundance of TLR was relative to 18S mRNA in the ileum of piglets offered a mid-transport lairage and piglets continuously transported. Trends for time effects for TLR2 ($P = 0.08$) and TLR5 ($P = 0.07$) were found.

contents compared with continuous pigs (Table 2). Lairage pigs had greater ($P < 0.05$) variation in microbial populations than continuous pigs and their cross products ($P < 0.05$) in cecal contents on d 3. Lairage pigs also had greater ($P < 0.01$) variation in cecal tissues compared with continuous pigs (Table 2). Lairage pigs had a lower ($P < 0.05$) similarity coefficient of jejunum contents compared with continuous pigs (Table 2) on d 7. A treatment effect ($P < 0.01$) was detected for jejunal and ileal tissue and cecal contents on d 14 (Table 2). In lairage pigs, a greater ($P = 0.01$) variation in microbial populations in jejunal tissue and cecal contents was observed. In ileal tissue, however, continuous pigs had greater ($P < 0.01$) variation.

DISCUSSION

Transportation is a significant stressor for most animals reared for food (Nabuurs et al., 2001; Broom, 2003, 2005; Odore et al., 2004; Warriss, 2004; Yagi et al., 2004). This stress has an effect not only on the immune system, but also on subsequent behaviors of pigs in an attempt to cope with the stressor (Warren et al., 1997; Zanella and Duran, 2001; Broom, 2006). Additionally, a deviation from normal immune function alters intestinal microbial populations during stressful events be-

cause of communication between the neuroendocrine system, gastrointestinal tract, commensal microflora, and the immune system (Ley et al., 2006). This study embedded a mid-journey lairage to determine if such an intervention could alter immune measures and intestinal microbial populations in response to a prolonged (16 h) transport.

One response to stress by the innate immune system is reflected in increased peripheral granulocytes, primarily neutrophils (Carlson et al., 1996; Yagi et al., 2004). Total peripheral circulating WBC counts, granulocyte numbers, and natural killer cell prevalence can nearly double during sympathetic nervous system activation. This drastic increase in number is due to alterations in WBC trafficking and the release of leukocytes from bone marrow resultant from elevated glucocorticoid concentration (Gupta et al., 2007). These and other types of responses aid in the ability of the piglet to cope with stressful events. In the present study, a mid-journey lairage reduced total WBC and granulocyte counts immediately following transport. This suggests that the amount of stress experienced by piglets was reduced despite the additional handling and on- and off-loading.

Immune system responses to transportation stress are important because the resulting suppression of de-

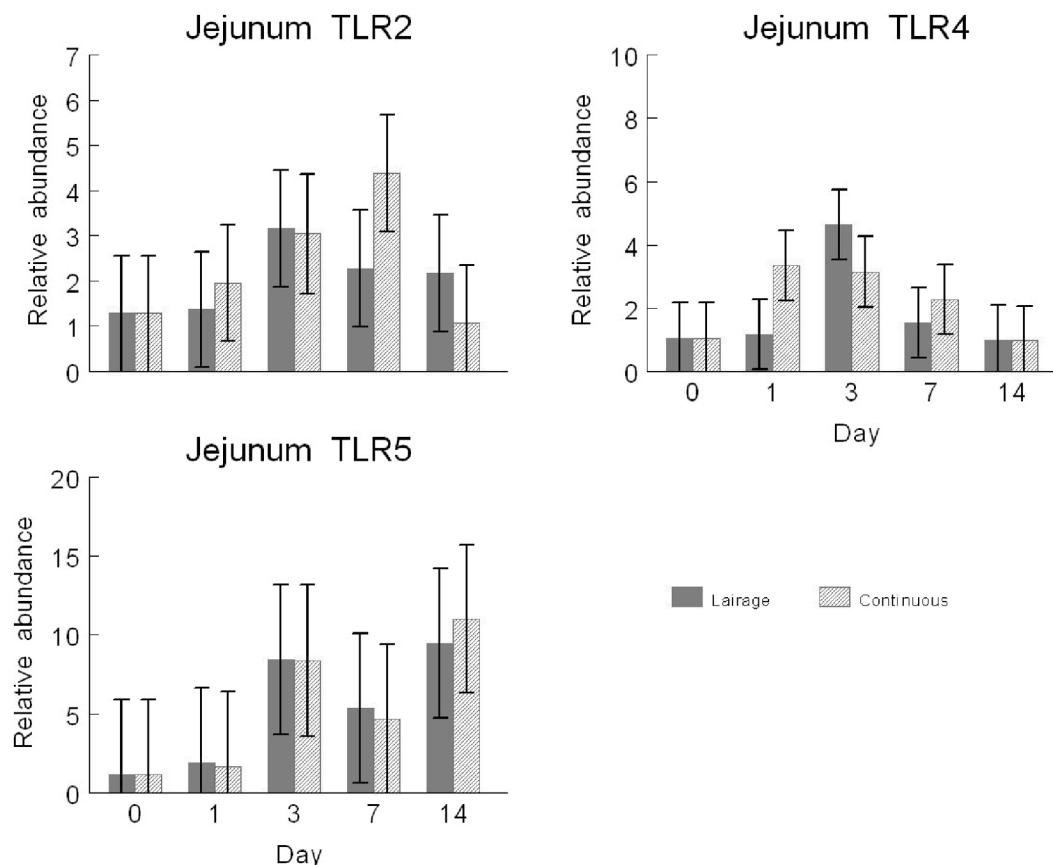


Figure 7. Relative abundance (least squares means \pm SE) of toll-like receptor (TLR) 2, TLR4, and TLR5 mRNA in jejunal tissues. Abundance of TLR was relative to 18S mRNA in the jejunum of piglets offered a mid-transport lairage and piglets continuously transported. Expression of TLR4 had a time effect ($P = 0.003$).

fense mechanisms increases the predisposition to disease, which can lead to significant productivity losses. Both CD14 and CD18 are important cell-surface molecules that recognize LPS on the surface of gram-negative bacteria and aid migration of leukocytes from peripheral blood to areas of inflammation, respectively (Troelstra et al., 1997; Janeway et al., 2001; Yagi et al., 2004). Porcine monocytes typically have high expression of CD14; however, upon differentiation into macrophages, downregulation of this surface molecule occurs resulting in a more specific macrophage surface marker (Basta et al., 1999). Our results indicate that with the addition of a lairage, neonatal pigs are better able to establish a mature population of phagocytes which have the potential to clear a pathogenic infection. Little research has been done to assess the role of physiological stress on cell adhesion molecules in swine. However, surface expression of CD18 by bovine neutrophils is reduced after 4 h of transport, but returns to basal levels 2 h after unloading. This may indicate that time points chosen in the current experiment may have been past the point of fluctuating expressions of CD18 due to transport stress (Yagi et al., 2004).

Toll-like receptor 4 is a pattern recognition receptor that recognizes LPS in conjunction with CD14 and is found on the surface of smooth muscle cells in the gut

as well as intestinal epithelial cells, macrophages, and dendritic cells found in the GI tract (Franchimont et al., 2004; Netea et al., 2004; Rumio et al., 2006). Because TLR4 is expressed on smooth muscle cells of the intestine, the jejunum, being the most muscular section of the GI tract, has more TLR4 expression than its counterparts (Rumio et al., 2006). The upregulation in TLR4 in the jejunum may be the reason for a time effect in the jejunum tissue of lairage pigs as opposed to other tissues. The antimicrobial peptide, PR39, was also more abundant in lairage pigs on d 3, possibly indicating that the piglets provided a lairage are inclined to have a better orchestrated immune response in the gut to maintain homeostasis between the GI epithelium, immune system, and commensal microbes. There are several suggested functions of PR39. Originally found as an anti-microbial peptide in the gut, it also has anti-nicotinamide-adenine dinucleotide phosphate oxidase properties which downregulates oxidative burst activities. Inhibiting respiratory burst is seemingly counter-intuitive, but perhaps PR39 also has a function as a regulator of gut microbial populations (Blecha, 2001).

Throughout the sampling period, with the exception of ileal tissue on d 14, the similarity coefficients of continuously transported piglets were greater than that of lairage piglets. This may indicate a greater amount of

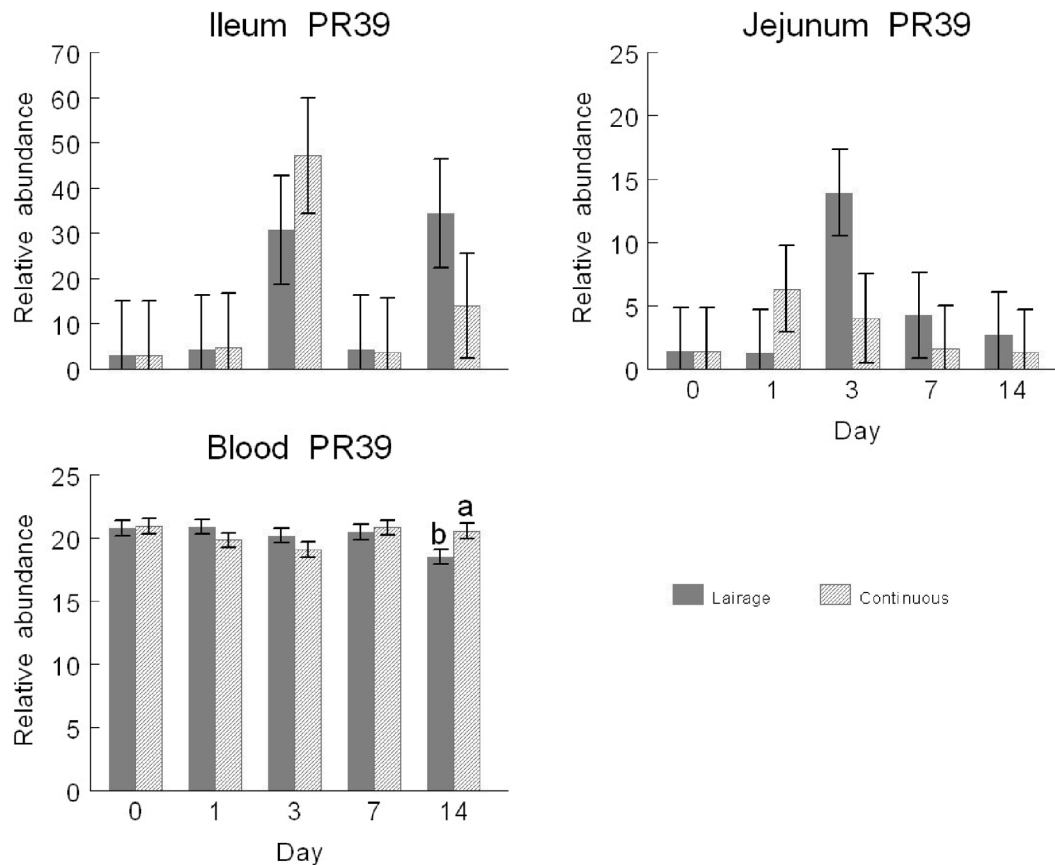


Figure 8. Relative abundance (least squares means \pm SE) of PR39 mRNA in ileal, jejunal, and blood tissues. Abundance of PR39 was relative to 18S mRNA in the ileum, jejunum, and blood of piglets offered a mid-transport lairage and piglets continuously transported. Jejunal PR39 expression had a time effect ($P < 0.01$) and expression of blood PR39 had a time effect ($P < 0.05$) and a treatment \times day effect ($P < 0.01$). ^{a,b}Means without common letters differ ($P < 0.05$).

Table 2. Similarity coefficients for microbial populations in jejunal, ileal, and cecal tissues and contents immediately after transport¹

Item	Jejunum		Ileum		Cecum	
	Contents	Tissue	Contents	Tissue	Contents	Tissue
Day 1						
Lairage ²	17.07 \pm 1.51 ^b	26.17 \pm 1.17	35.91 \pm 1.6	30.00 \pm 3.37	46.49 \pm 1.38	28.14 \pm 1.50
Continuous ²	24.51 \pm 1.60 ^a	26.06 \pm 1.15	40.80 \pm 1.56	35.87 \pm 3.4	44.00 \pm 1.38	30.81 \pm 1.42
Cross product ³	20.89 \pm 1.09 ^{ab}	26.83 \pm 0.82	39.05 \pm 1.11	33.25 \pm 2.36	45.24 \pm 0.97	30.37 \pm 1.03
Day 3						
Lairage ²	19.58 \pm 1.16	30.75 \pm 1.39	35.00 \pm 2.03	24.66 \pm 1.73	43.64 \pm 0.94 ^b	29.90 \pm 0.96 ^b
Continuous ²	22.55 \pm 1.36	32.12 \pm 1.39	38.78 \pm 2.03	30.11 \pm 1.73	48.72 \pm 0.95 ^a	34.53 \pm 0.93 ^a
Cross product ³	20.90 \pm 0.87	32.27 \pm 0.98	36.97 \pm 1.43	28.44 \pm 1.30	46.56 \pm 0.67 ^a	32.60 \pm 0.66 ^{ab}
Day 7						
Lairage ²	18.60 \pm 1.21 ^b	37.90 \pm 1.37	35.47 \pm 1.44	28.79 \pm 1.30	43.21 \pm 2.39	41.35 \pm 1.27
Continuous ²	23.55 \pm 1.36 ^a	33.38 \pm 1.36	35.68 \pm 1.39	28.79 \pm 1.17	46.24 \pm 2.39	44.05 \pm 1.26
Cross product ³	19.74 \pm 0.89 ^{ab}	34.23 \pm 0.96	37.37 \pm 0.99	29.82 \pm 0.85	45.93 \pm 1.65	42.64 \pm 0.89
Day 14						
Lairage ²	21.68 \pm 1.18	34.81 \pm 1.32 ^b	35.14 \pm 1.85	30.09 \pm 1.12 ^a	49.36 \pm 1.81 ^b	38.02 \pm 1.47
Continuous ²	21.25 \pm 1.15	40.99 \pm 1.33 ^a	34.89 \pm 1.83	24.19 \pm 1.10 ^b	57.52 \pm 1.85 ^a	39.82 \pm 1.45
Cross product ³	21.77 \pm 0.82	38.22 \pm 0.93 ^{ab}	35.29 \pm 1.30	26.89 \pm 0.77 ^{ab}	52.43 \pm 1.29 ^{ab}	38.02 \pm 1.03

^{a,b}Means within a column without common superscripts differ ($P < 0.01$).

¹Values represent least squares means \pm SE.

²Within-treatment similarity coefficients for microbial populations in the intestine of piglets offered a mid-transport lairage and piglets continuously transported.

³Across-treatment similarity coefficients for microbial populations; comparisons between each individual pig and all the other individuals of the opposite treatment.

stress imposed on these piglets by prolonged transportation. Variability of bacterial communities may be caused either by inhibition of some predominant bacteria or enhancement of the growth of specific bacterial species to the point that they dominate the intestinal microbiota. Several studies have indicated that external stressors decrease the variability of the microbiota present in the small intestine. Even short-duration transportation (30 min) can induce small intestinal acidosis in swine leading to a reduced population of acid-compatible bacteria (Nabuurs et al., 2001). Additionally, physiologically stressful events lead to an increase in neurohormone concentration in the intestinal tract which affects gastrointestinal functioning. For example, norepinephrine slows intestinal transit (Bailey et al., 1999), increases adherence of the highly pathogenic *E. coli* O157:H7 to porcine colonic tissue and enhances growth of LPS-containing gram-negative bacteria leading to well-colonized overgrowths of one type of bacteria (Bailey et al., 1999; Green et al., 2004). Additionally, this difference between treatments may have been exacerbated by the timing of access to feed and water. Both treatments had equal access to feed and water in a 24-h period, but at different times of the day and at different times relative to transport. Interactions of the gut and the immune system are imperative for adequate protection of the host from both commensal bacteria and pathogens otherwise pathology results (McCracken and Lorenz, 2001).

Data in this study suggest that prolonged transport of 18-kg pigs with a lairage including access water and to food for 2 h alters immune system responses after the transport. These alterations encompass peripheral immune cell populations and expression of cell surface recognition and adhesion molecules, and an antimicrobial peptide in the gut. Changes in the immune system responses lead to shifts in gut microbial populations, and fewer populations of microbiota may ultimately result in pathogenesis.

LITERATURE CITED

- Bailey, M. T., J. W. Karaszewski, G. R. Lubach, C. L. Coe, and M. Lyte. 1999. In vivo adaptation of attenuated *Salmonella typhimurium* results in increased growth upon exposure to norepinephrine. *Physiol. Behav.* 67:359–364.
- Basta, S., S. M. Knoetig, M. Spagnuolo-Weaver, G. Allan, and K. C. McCullough. 1999. Modulation of monocytic cell activity and virus susceptibility during differentiation into macrophages. *J. Immunol.* 162:3961–3969.
- Blecha, F. 2001. *Immunology. Biology of the domestic pig*. Cornell University Press, Ithaca, NY.
- Broom, D. M. 2003. Causes of poor welfare in large animals during transport. *Vet. Res. Commun.* 27(Suppl. 1):515–518.
- Broom, D. M. 2005. The effects of land transport on animal welfare. *Rev. Sci. Tech.* 24:683–691.
- Broom, D. M. 2006. Behaviour and welfare in relation to pathology. *Appl. Anim. Behav. Sci.* 97:73–83.
- Carlson, S. L., D. J. Beiting, C. A. Kiani, K. M. Abell, and J. P. McGillis. 1996. Catecholamines decrease lymphocyte adhesion to cytokine-activated endothelial cells. *Brain Behav. Immun.* 10:55–67.
- Franchimont, D., S. Vermeire, H. El Housni, M. Pierik, K. Van Steen, T. Gustot, E. Quertinmont, M. Abramowicz, A. Van Gossom, J. Deviere, and P. Rutgeerts. 2004. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (tlr)-4 asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut* 53:987–992.
- Green, B. T., M. Lyte, C. Chen, Y. Xie, M. A. Casey, A. Kulkarni-Narla, L. Vulchanova, and D. R. Brown. 2004. Adrenergic modulation of *Escherichia coli* O157:H7 adherence to the colonic mucosa. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287:G1238–G1246.
- Gupta, S., B. Earley, and M. A. Crowe. 2007. Effect of 12-hour road transportation on physiological, immunological and haematological parameters in bulls housed at different space allowances. *Vet. J.* 173:594–604.
- Janeway, C. A., P. Travers, M. Walport, and M. Shlomchik. 2001. *Immunobiology*. 5th ed. Garland Publishing, New York, NY.
- Ley, R. E., D. A. Peterson, and J. I. Gordon. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124:837–848.
- McCracken, V. J., and R. G. Lorenz. 2001. The gastrointestinal ecosystem: A precarious alliance among epithelium, immunity and microbiota. *Cell. Microbiol.* 3:1–11.
- Nabuurs, M. J., G. J. Van Essen, P. Nabuurs, T. A. Niewold, and J. Van Der Meulen. 2001. Thirty minutes transport causes small intestinal acidosis in pigs. *Res. Vet. Sci.* 70:123–127.
- Netea, M. G., C. van der Graaf, J. W. Van der Meer, and B. J. Kullberg. 2004. Toll-like receptors and the host defense against microbial pathogens: Bringing specificity to the innate-immune system. *J. Leukoc. Biol.* 75:749–755.
- Odore, R., A. D'Angelo, P. Badino, C. Bellino, S. Pagliasso, and G. Re. 2004. Road transportation affects blood hormone levels and lymphocyte glucocorticoid and beta-adrenergic receptor concentrations in calves. *Vet. J.* 168:297–303.
- Richlin, V. A., J. M. Arevalo, J. A. Zack, and S. W. Cole. 2004. Stress-induced enhancement of nf-kb DNA-binding in the peripheral blood leukocyte pool: Effects of lymphocyte redistribution. *Brain Behav. Immun.* 18:231–237.
- Rumio, C., D. Besusso, F. Arnaboldi, M. Palazzo, S. Selleri, S. Garioldi, S. Akira, S. Uematsu, P. Bignami, V. Ceriani, S. Menard, and A. Balsari. 2006. Activation of smooth muscle and myenteric plexus cells of jejunum via toll-like receptor 4. *J. Cell. Physiol.* 208:47–54.
- Soderholm, J. D., D. A. Yates, M. G. Gareau, P. C. Yang, G. MacQueen, and M. H. Perdue. 2002. Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. *Am. J. Physiol. Gastrointest. Liver Physiol.* 283:G1257–G1263.
- Troelstra, A., B. N. Giepmans, K. P. Van Kessel, H. S. Lichenstein, J. Verhoef, and J. A. Van Strijp. 1997. Dual effects of soluble CD14 on LPS priming of neutrophils. *J. Leukoc. Biol.* 61:173–178.
- Warren, E. J., B. N. Finck, S. Arkins, K. W. Kelley, R. W. Scamurra, M. P. Murtaugh, and R. W. Johnson. 1997. Coincidental changes in behavior and plasma cortisol in unrestrained pigs after intracerebroventricular injection of tumor necrosis factor- α . *Endocrinology* 138:2365–2371.
- Warriss, P. D. 2004. The transport of animals: A long way to go. *Vet. J.* 168:213–214.
- Yagi, Y., H. Shiono, Y. Chikayama, A. Ohnuma, I. Nakamura, and K.-I. Yayou. 2004. Transport stress increases somatic cell counts in milk, and enhances the migration capacity of peripheral blood neutrophils of dairy cows. *J. Vet. Med. Sci.* 66:381–387.
- Zanella, A. J., and O. Duran. 2001. Pig welfare during loading and transportation: A North American perspective. Pages 20–31 in 1st Int. Virtual Conf. on Pork Quality—Welfare, Transport and Consumer. Embrapa Publ., Concórdia, Brazil.

References

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